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Tumorigenesis and Neoplastic Progression

Interleukin-11 in Endometrial Adenocarcinoma Is Regulated by Prostaglandin F_{2α}-F-Prostanoid Receptor Interaction via the Calcium-Calcineurin-Nuclear Factor of Activated T Cells Pathway and Negatively Regulated by the Regulator of Calcineurin-1

Kurt J. Sales,* Vivien Grant,* Ian H. Cook,*
David Maldonado-Pérez,**† Richard A. Anderson,†
Alistair R.W. Williams,‡ and Henry N. Jabbour*

From the Medical Research Council Human Reproductive Sciences Unit,* and the Departments of Reproductive and Developmental Sciences,† and Pathology,‡ The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom

Interleukin-11 (IL-11) up-regulates the proliferative and invasive capacity of many cancers. Coexpression of glycoprotein 130 (GP130) and IL-11 receptor α (IL-11R α) is necessary for high-affinity binding of IL-11 to IL-11R α . This study investigated the expression of IL-11 and role of prostaglandin F_{2α}-F-prostanoid receptor (FP receptor) signaling in the modulation of IL-11 expression in endometrial adenocarcinoma cells. Localization of IL-11, IL-11R α , and GP130 expression was performed by immunohistochemistry. IL-11 and regulator of calcineurin 1 isoform 4 (RCAN1-4) mRNA and protein expression were determined by real-time RT-PCR and/or enzyme-linked immunosorbent assay/Western blot analysis using Ishikawa endometrial adenocarcinoma cells stably expressing the FP receptor (FPS cells) and endometrial adenocarcinoma explants. IL-11 mRNA expression was significantly elevated in endometrial adenocarcinoma samples compared with normal endometrium and increased with tumor grade. IL-11 protein expression localized with FP receptor, IL-11R α , and GP130 in the neoplastic glandular epithelium of endometrial adenocarcinomas. Prostaglandin F_{2α}-FP receptor signaling significantly elevated the expression of IL-11 mRNA and protein in a Gq-protein kinase C-calcium-calcineurin-nuclear factor of activated T cells-dependent manner

in FPS cells. The calcineurin signaling pathway is known to be controlled by the RCAN (RCAN1-4). Indeed, RCAN1-4 expression was significantly elevated in well-differentiated endometrial adenocarcinoma compared with normal endometrium and was found to decrease with tumor grade and negatively regulate IL-11 expression *in vitro*. This study has highlighted a new mechanism regulating IL-11 expression in endometrial adenocarcinoma cells by the FP receptor via the calcium-calcineurin-nuclear factor of activated T cells pathway. (Am J Pathol 2010, 176:435–445; DOI: 10.2353/ajpath.2010.090403)

Endometrial cancer is the most common female gynecological malignancy in the Western world, ranking fourth in incidence among invasive tumors in women.^{1–3} Most cases of endometrial carcinomas are sporadic estrogen-dependent disorders that occur in pre- or postmenopausal women as low-grade (well differentiated, type I) endometrioid adenocarcinomas.⁴ However, ~20% of tumors in postmenopausal women are not estrogen dependent and have a poor prognosis.^{1–4} In these patients, predominantly high-grade (poorly differentiated, type II) tumors arise either as endometrioid adenocarcinomas, uterine papillary serous carcinomas, or clear cell carcinomas with a high frequency of myometrial invasion and spread into the pelvic lymph nodes.⁵

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Address reprint requests to Henry N. Jabbour, Ph.D., Human Reproductive Sciences Unit, Medical Research Council, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. E-mail: h.jabbour@hrc.mrc.ac.uk.

Although the mechanisms regulating endometrial adenocarcinomas are still poorly defined, there is much evidence for a role for cyclooxygenase (COX) enzymes and prostaglandins (PGs) in uterine pathology. We and others^{6,7} have demonstrated elevated expression of COX-2, biosynthesis of PG, and elevated expression of nuclear⁶ and membrane-bound G protein-coupled receptors^{6,8} like the F-prostanoid (FP) receptor⁸ in endometrial adenocarcinomas. Moreover, we have shown that elevated $\text{PGF}_{2\alpha}$ -FP receptor signaling, via the Gq activation of inositol-1,4,5-trisphosphate, leads to up-regulation of tumorigenic and angiogenic genes including COX-2,⁹ fibroblast growth factor 2,¹⁰ and vascular endothelial growth factor,¹¹ indicating that $\text{PGF}_{2\alpha}$ -FP receptor signaling can promote endometrial tumor growth by regulating vascular function. Furthermore, FP receptor can regulate the proliferation of endometrial epithelial cells and can alter their adhesiveness to extracellular matrix and motility via the reorganization of the actin cytoskeleton and activation of focal adhesion kinase.^{8,12,13} These findings suggest that $\text{PGF}_{2\alpha}$ -FP receptor signaling plays a multifactorial role in regulating endometrial adenocarcinoma by promoting an environment for angiogenesis and tissue remodeling to facilitate tumor growth.

In addition to the regulation of cell architecture and growth factors by the COX-PG axis, a link between PG and chemoattractive cytokines (chemokines) such as CXCL1 has been demonstrated in colorectal cancer,¹⁴ where PGE_2 signaling has been shown to induce CXCL1 expression in colorectal cancer cells to enhance tumor growth. Similarly in COX-2-overexpressing breast cancer cells that had metastasized to bone, Singh et al¹⁵ have shown recently that the pleiotropic cytokine interleukin-11 (IL-11) is significantly elevated.¹⁵ IL-11 mediates its function via the IL-11 receptor α (IL-11R α). On ligand binding to IL-11R α , the glycoprotein (GP) 130 subunit, critical for signal transduction of IL-11, is recruited to form a IL-11/IL-11R α /GP130 complex.¹⁶ Once activated, the IL-11/IL-11R α /GP130 complex can activate signal transduction pathways¹⁷ to modulate target gene expression. IL-11 and IL-11R α expression has been shown to correlate with cellular growth, differentiation, invasiveness, tumor progression, and poor prognosis in breast and colorectal cancer;^{18–20} however, the expression and regulation of IL-11 in endometrial cancer has yet to be reported.

Here we investigated the expression profile of IL-11, IL-11R α , and GP130 in endometrial adenocarcinomas compared with normal proliferative-phase endometrium and its regulation in an *in vitro* model of endometrial adenocarcinoma cells by $\text{PGF}_{2\alpha}$ via the FP receptor.

Materials and Methods

Reagents

YM-254890 was donated by Astellas Pharma (Tsukuba, Japan). Cyclosporin A and Inhibitor of nuclear factor of activated T cells (NFAT)-calcineurin association-6 (Inca-6), 4-cyano-3-methylisoquinoline, and RO-318220 were purchased from Calbiochem (Nottingham, UK). $\text{PGF}_{2\alpha}$,

AL8810, and EGTA were purchased from Sigma Chemical (Dorset, UK).

Cell Line, Culture, and Treatments

Ishikawa cells engineered to stably express the full-length human FP (PTGFR, accession no. NM_000959) receptor to the levels observed in endometrial adenocarcinomas, referred to as FPS cells, were cultured as described previously.¹¹ FPS cells were manufactured commercially, clonally selected, and verified as described in our previous study.¹¹ The concentrations of all chemical inhibitors and antibodies were determined empirically by titration using the manufacturer's guidelines as described in our previous studies.^{21,22} Cell viability in the presence of chemical inhibitor was determined using the CellTiter 96 Aqueous One Solution assay (Promega, Southampton, UK) as described previously.²² Cells were treated with 100 nmol/L $\text{PGF}_{2\alpha}$ alone or in the presence of AL8810 (50 $\mu\text{mol/L}$), YM254890 (1 $\mu\text{mol/L}$), 43CMQ (1 $\mu\text{mol/L}$), RO-318220 (1 $\mu\text{mol/L}$), AG1478 (200 nmol/L), cyclosporine A (1 $\mu\text{mol/L}$), Inca-6 (40 $\mu\text{mol/L}$), and EGTA (1.5 mmol/L) for the time indicated. In parallel, cells treated with vehicle and chemical inhibitor served as a control for each treatment. Fold increase was calculated by dividing the values obtained from the $\text{PGF}_{2\alpha}$ / $\text{PGF}_{2\alpha}$ -inhibitor treatments by the vehicle/vehicle-inhibitor treatments. All *in vitro* cell culture experiments were performed in duplicate.

Human Tissue

Poorly differentiated (grade 3; $n = 10$), moderately differentiated (grade 2; $n = 10$), and well-differentiated (grade 1; $n = 10$) endometrial adenocarcinoma tissues with clinical characteristics as outlined in Table 1 were collected from women undergoing hysterectomy and who had been pre-diagnosed on endometrial biopsy to have endometrial adenocarcinoma of the uterus of the endometrioid type. The patients had received no treatment before surgery. All patients were postmenopausal women with ages that ranged between 50 and 71 years of age and presented with complaint of postmenopausal bleeding. The median age of all patients in our study was 60.5 years. Total abdominal hysterectomy specimens with bilateral salpingo-oophorectomy for adenocarcinoma were collected from the operating theater and placed on ice. With minimal delay, the specimens were opened by a gynecological pathologist. Small samples (~5 mm to 3 cm) of polypoidal adenocarcinoma tissue were collected from the endometrial lumen. Tissue samples were transferred into neutral-buffered formalin (for paraffin wax embedding for immunohistochemistry and immunofluorescence studies), snap-frozen in dry ice and stored at -70°C (for RNA extraction), and placed in RPMI 1640 culture medium containing 2 mmol/L L-glutamine, 100 U of penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 8.4 $\mu\text{mol/L}$ indomethacin (to inhibit endogenous COX activity) for *ex vivo* explant cultures. The diagnosis of endometrial adenocarcinoma was confirmed histologically in all cases as defined in Table 1

Table 1. Clinical Parameters and Tumor Characteristics for Poorly (Poor, *n* = 10), Moderately (Mod, *n* = 10), and Well-Differentiated (Well, *n* = 10) Endometrial Adenocarcinomas

Sample no.	Grade	FIGO stage
1	Poor	Ia
2	Poor	IIIa
3	Poor	Ib
4	Poor	Ib
5	Poor	IIIa
6	Poor	Ib
7	Poor	Ib
8	Poor	IIIa
9	Poor	IIIa
10	Poor	IIb*
11	Mod	Ib
12	Mod	Ic
13	Mod	IIb*
14	Mod	IIIa
15	Mod	Ic
16	Mod	Ic
17	Mod	Ic
18	Mod	Ia
19	Mod	Ib
20	Mod	IIb
21	Well	Ib
22	Well	Ib
23	Well	Ia
24	Well	Ic
25	Well	IIIa
26	Well	Ib
27	Well	Ib
28	Well	Ib
29	Well	Ia
30	Well	Ia

All endometrial adenocarcinomas studied were of pure or predominantly of endometrioid type. There were no pure serous or clear cell subtypes in the series, and none of the tumors showed carcinosarcoma.* In these two samples myometrial invasion was <50%.

and the percentage of tumor cells to stroma was estimated to be not less than 75%:25%. Normal endometrium from the proliferative phase of the menstrual cycle, with clinical parameters as outlined in Table 2 (*n* = 10), was collected with an endometrial suction curette from women undergoing surgery for gynecological procedures including surgical sterilization or abnormal uterine bleeding in whom histological examination of endometrium was normal with no underlying endometrial pathology (Pipelle, Laboratoire CCD, Paris, France). This phase of the

Table 2. Clinical Parameters for Normal Endometrial Samples from Proliferative-Phase Endometrium (*n* = 10)

Sample no.	Histology	Progesterone (nmol/L)	Estradiol (pmol/L)
1	Proliferative	4.26	339.38
2	Proliferative	2.22	641.37
3	Proliferative	4.62	525.00
4	Proliferative	4.57	495.00
5	Proliferative	2.82	214.00
6	Proliferative	1.78	400.00
7	Proliferative	4.80	204.00
8	Proliferative	3.12	731.00
9	Proliferative	2.32	1796.00
10	Proliferative	0.17	989.70

menstrual cycle was chosen as a comparator for the endometrial cancer samples as it exhibits all of the hallmarks of endometrial tumorigenesis, namely low progesterone levels, rapid cellular proliferation, differentiation and tissue remodeling and is the phase of the menstrual cycle with the highest expression of FP receptor.¹³ All women had regular menstrual cycles (25 to 35 days) and the tissue collected was processed exactly as described above. The ages of the control women ranged from 21 to 39 years of age with a median age of 30.5 years. None of the control women had received a hormonal preparation in the 3 months preceding biopsy collection. Biopsies were dated according to stated last menstrual period and confirmed by histological assessment according to the criteria of Noyes et al.²³ Ethical approval was obtained from Lothian Research Ethics Committee, and written informed consent was obtained from all subjects before tissue collection.

TaqMan Quantitative RT-PCR

Total RNA was extracted from FPS cells and proliferative-phase endometrium (*n* = 10; samples 1 to 10 in Table 2), poorly differentiated (*n* = 10; samples 1 to 10 in Table 1), moderately differentiated (*n* = 10; samples 11 to 20 in Table 1), and well-differentiated (*n* = 10; samples 21 to 30 in Table 1) adenocarcinoma tissues using Total RNA Isolation Reagent (Abgene, Epsom, UK), according to the manufacturer's instruction. Tissue lysis was performed using a tissue lyser (Qiagen, Crawley, UK). Quantified RNA samples were reverse transcribed, and quantitative RT-PCR was performed as described before⁸ using the following primers and probes: IL-11, forward, 5'-CCCAGTTACCCAAGCATCCA-3', and reverse, 5'-AGACAGAGAACAGGGAATTAATGTGT-3', and probe, 5'-FAM-CCCCAGCTCTCAGACAAATCGCCC-3'; IL-11Rα, forward, 5'-CCAGCCAGATCAGCGGTTTA-3', and reverse, 5'-TGGCTATCAGCTCCTAGGACTGT-3', and probe, 5'-FAM-CCACCCGCTACCTCACCTCCTACAGG-3'; and GP130, forward, 5'-CTGAATGGGCAACACA-CAAGTT-3', and reverse, 5'-CCAGACTTCAATGTTGACAAAATACA-3', and probe, 5'-FAM-CAAAGCAAAA-CGTGACACCCCCACC-3'. Primers and data were analyzed and processed using Sequence Detector version 1.6.3 (Applied Biosystems, Warrington, UK). Expression of analyzed genes was normalized to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed either as relative mRNA expression or as fold increase above vehicle-treated cells. Data are presented as mean ± SEM. Cell culture experiments were repeated three times in duplicate.

Immunohistochemistry

Immunohistochemistry for IL-11, IL-11Rα, and GP130 was performed using poorly (*n* = 10; samples 1 to 10 in Table 1), moderately (*n* = 10; samples 11 to 20 in Table 1), and well-differentiated (*n* = 10; samples 21 to 30 in Table 1) endometrial adenocarcinomas and proliferative-phase endometrium (*n* = 10; Table 2) using the Vision Biosys-

tems Bond Immunostaining Robot (Leica Microsystems, Wetzlar, Germany) as described previously.^{7,8,10} Control sections were incubated with equivalent concentration of normal IgG from host species.

Immunofluorescence and Confocal Laser Microscopy

Colocalization of FP receptor, IL-11R α , and IL-11 protein expression was investigated using a random selection of tissues from patient samples as outlined in Tables 1 and 2, comprising normal proliferative ($n = 5$; samples 1 to 5 in Table 2) and well-differentiated endometrial cancer tissues ($n = 4$; samples 21 to 24 in Table 1). Five-micrometer paraffin wax-embedded tissue sections were cut and mounted onto coated slides. Sections were dewaxed in xylene, rehydrated in graded ethanol, and washed in water. Antigen retrieval was performed by pressure cooking the slides for 5 minutes in 0.01 M sodium citrate (pH 6). After antigen retrieval, endogenous endoperoxidase activity was blocked by incubating slides in methanol containing 3% H₂O₂. For the triple immunofluorescence, sections were blocked in normal goat serum (NGS) and incubated overnight with a polyclonal anti-IL-11R α antibody at 1/600 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed, incubated in goat anti-rabbit peroxidase (1/500 in NGS for 30 minutes), followed by fluorochromes tissue specific antigen-plus cyanide 3 (1/50 in diluent for 10 minutes; PerkinElmer, Wellesley, MA; Applied Biosystems). Sections were washed, and antigen retrieval in citrate buffer and blocking in NGS was repeated before being incubated overnight with a polyclonal anti-FP receptor antibody at 1/600 (Cayman Chemical, Ann Arbor, MI). Sections were washed, incubated in goat anti-rabbit peroxidase (1/500 in NGS), followed by fluorochromes tissue specific antigen-plus cyanide 5 (1/50 in diluent). Finally, after further antigen retrieval and blocking steps, sections were incubated overnight with a monoclonal anti-IL-11 antibody at 1/100 (R&D Systems, Oxford, UK). Sections were washed, incubated in goat anti-mouse peroxidase (1/500 in NGS), followed by fluorochromes tissue specific antigen-plus fluorescein (green) (1/50 in diluent). All sections were washed, mounted in Permafluor, and visualized using a laser-scanning microscope (meta confocal; Carl Zeiss, Jena, Germany). Matched serial control sections from each tissue were incubated with equivalent concentration of normal IgG from host species.

Enzyme-Linked Immunosorbent Assay

Secreted IL-11 was quantified using an in-house enzyme-linked immunosorbent assay (ELISA). IL-11 was measured by ELISA using matched pairs of capture and biotinylated labeled detection antibodies for IL-11 (R&D Systems). Briefly, plates were coated overnight at 4°C with capture antibody added at 100 μ l/well, followed by 100 μ l/well of coating solution for 1 hour at room temperature. The coating solution was removed and the plates

stored at -20°C. Before use, the plates were washed twice in wash buffer (0.05% Tween 20, 10 mmol/L Tris, and 0.15 M NaCl). Recombinant IL-11 standard (R&D Systems) was diluted in ELISA buffer (using a concentration range of 4000 to 31.25 pg/ml), and 100 μ l of standard or conditioned media sample was added per well in duplicate. Plates were incubated overnight at 4°C and then washed four times as above, before addition of anti-IL-11 detection antibody (R&D Systems) at 100 ng/ml in ELISA buffer. Plates were again washed four times before addition of streptavidin-peroxidase at 1/2000 adding 100 μ l/well for 20 minutes at room temperature on a rocker. Plates were again washed four times before addition of tetramethylbenzidine substrate. Color change was monitored and stopped using 2 N sulfuric acid. Plates were read on a plate reader at 450 nm within 10 minutes of quenching. Samples were serially diluted before assay, and the concentration of IL-11 in each sample was interpolated from the within plate standard curve. The minimum detection limit was 16 pg/ml. The interassay and intra-assay coefficient of variance were 7.31 and 6.04%, respectively. Data are represented as mean \pm SEM for four individual experiments.

Western Blot Analysis

Proteins were extracted and quantified as described previously.⁸ Samples were loaded in duplicate. In parallel, cell lysate from Ishikawa cells infected with full-length regulator of calcineurin 1-4 (RCAN1-4) adenovirus as described by Maldonado-Perez et al²¹ was used as a positive control (data not shown). After resolving and immunoblotting, membranes were incubated overnight at 4°C, with a rabbit anti RCAN1-4 antibody (1/5000), a gift from Dr. E. W. Bush (Myogen, Westminster, CO), together with a mouse anti- β -actin antibody (1/800) (Santa Cruz Biotechnology). The following day, cells were washed and incubated with goat anti-rabbit Alexafluor 680 (1/5000; Invitrogen, Carlsbad, CA) and goat anti-mouse IRDye 800 (1/5000; Rockland, Gilbertsville, PA) for 60 minutes at room temperature. Blots were visualized and the protein immunoreactivity quantified using an Odyssey infrared imaging system (LI-COR, Cambridge, UK). RCAN1-4 relative density was calculated by dividing the value obtained for RCAN1-4 by the value obtained for β -actin and expressed as fold above vehicle controls. Data are presented as mean \pm SEM from three independent experiments.

Adenovirus Infection

The RCAN adenovirus was constructed using the cDNA of RCAN1-4 (Origene, Rockville, MD) as detailed in our previous study.²¹ Ishikawa FPS cells were plated in 5-cm dishes or 6-well plates at a density of 200,000 cells/well. Thereafter, cells were infected with RCAN1-4 adenovirus at a multiplicity of infection of five viruses per cell. In parallel, cells were infected with a negative control adenovirus containing a cytomegalovirus promoter with no insert (Rad60)²⁴ at the same multiplicity of infection. For

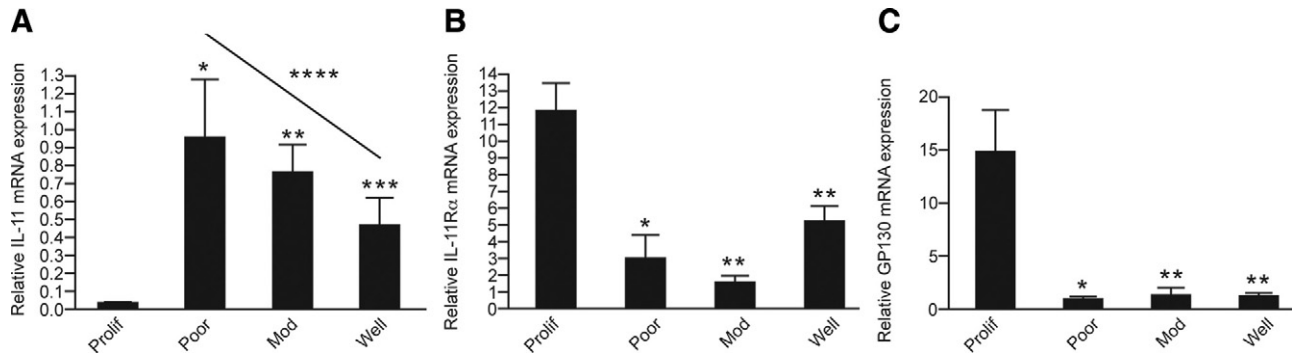


Figure 1. IL-11, IL-11R α , and GP130 mRNA expression. **A:** The relative mRNA expression of IL-11 in normal proliferative-phase endometrium ($n = 10$) and poorly ($n = 10$), moderately ($n = 10$), and well-differentiated ($n = 10$) endometrial adenocarcinoma as determined by real-time quantitative RT-PCR analysis. Data are represented as mean \pm SEM proliferation is significantly different from poorly ($*P < 0.001$), moderately ($**P = 0.005$), and well-differentiated ($***P = 0.02$) adenocarcinomas compared with proliferative-phase endometrium; ANOVA posttest for linear trend for IL-11 expression between poorly, moderately, and well-differentiated adenocarcinomas, $****P < 0.0001$. The relative mRNA expression of IL-11R α (**B**) and GP130 (**C**) in normal proliferative-phase endometrium ($n = 10$) and poorly ($n = 10$), moderately ($n = 10$), and well-differentiated ($n = 10$) endometrial adenocarcinoma as determined by real-time quantitative RT-PCR analysis. Data are represented as mean \pm SEM proliferation is significantly different from poorly ($*P = 0.03$), moderately, and well-differentiated ($**P = 0.04$) adenocarcinomas compared with proliferative-phase endometrium.

RCAN infection of endometrial adenocarcinoma tissues, four poorly differentiated tissues (samples 1 to 4; Table 1) were used. The tissues were processed in the order in which they were received from the gynecology clinic without bias toward clinical parameters such as International Federation of Gynecology and Obstetrics (FIGO) stage or depth of invasion. Tissues were prepared for infection by finely sectioning using a sterile scalpel blade and incubated with 1×10^6 virus/ml of either the RAD60 or RCAN1-4 adenovirus. Cells and tissues were incubated with virus for 24 hours and serum starved overnight before treatment with vehicle or 100 nmol/L PGF_{2α}. All infections and treatments were conducted in duplicate. Data are presented as mean \pm SEM (from four independent experiments for in vitro cell culture and $n = 4$ for adenocarcinoma explants).

Statistical Analysis

The data in this study were analyzed by unpaired *t*-test or analysis of variance (ANOVA) to compare groups using Microsoft Excel (Microsoft) and Prism 4.0c (GraphPad, San Diego, CA) with an additional posttest to assess the evidence of a linear trend across grades of adenocarcinomas where appropriate. Values of $P < 0.05$ were considered significant.

Results

IL-11, IL-11R α , and GP130 Expression in Endometrial Adenocarcinoma and Normal Proliferative-Phase Endometrium

The relative mRNA expression of IL-11 (Figure 1A), its receptor IL-11R α (Figure 1B), and its coreceptor GP130 (Figure 1C) was investigated in human endometrial adenocarcinoma and normal proliferative-phase endometrium by TaqMan quantitative RT-PCR analysis. The expression of IL-11 mRNA (Figure 1A) was significantly up-regulated in all endometrial adenocarcinomas repre-

sented in Table 1 irrespective of grade or FIGO stage, compared with normal proliferative-phase endometrial tissues listed in Table 2 (*t*-test, prolif versus Poor $P < 0.001$; *t*-test, prolif versus Mod $P = 0.005$; *t*-test, prolif versus well $P = 0.02$). In addition, a significant linear trend was observed across the different grades of cancer for IL-11 mRNA with highest levels of expression observed in poorly differentiated cancer (*, ANOVA posttest for linear trend; $P < 0.0001$). Expression of IL-11R α (Figure 1B) and GP130 (Figure 1C) was significantly decreased in all endometrial adenocarcinomas represented in Table 1 compared with normal proliferative-phase endometrium listed in Table 2 (*t*-test, prolif versus Poor $P = 0.03$; *t*-test, prolif versus Mod $P = 0.04$; *t*-test, prolif versus well $P = 0.04$) and did not appear to vary with tumor grade or FIGO stage.

The site of IL-11, IL-11R α , and GP130 protein expression in endometrial adenocarcinomas was determined by immunohistochemistry (Figure 2). Strong apical and baso-lateral immunoreactive staining was observed for IL-11 (Figure 2, A, D, and G), IL-11R α (Figure 2, B, E, and H), and GP130 (Figure 2, C, F, and I) in the glandular epithelium and vascular endothelium (BV, Figure 2, M–O, as indicated by the arrow; representative sample 29; Table 1) of poorly differentiated (representative sample 2; Table 1), moderately differentiated (Mod, representative sample 15; Table 1), and well-differentiated (representative sample 26; Table 1) endometrial adenocarcinomas with stromal staining observed for all three biomarkers. The expression patterns of IL-11, IL-11R α , and GP130 were similar within each respective grade irrespective of clinical parameter outlined in Table 1. The pattern of staining for IL-11, IL-11R α , and GP130 was similar in endometrial adenocarcinomas to that observed for normal proliferative endometrium (Figure 2, J–L). IL-11 localized to the apical surface (dark arrow) with punctuate lateral and basal (white arrow) surface staining (Figure 2J, representative sample 2; Table 2) in proliferative-phase endometrium with low levels of staining observed for IL-11 in the stroma. IL-11R α exhibited higher intensity of staining on the apical (dark arrow) compared with

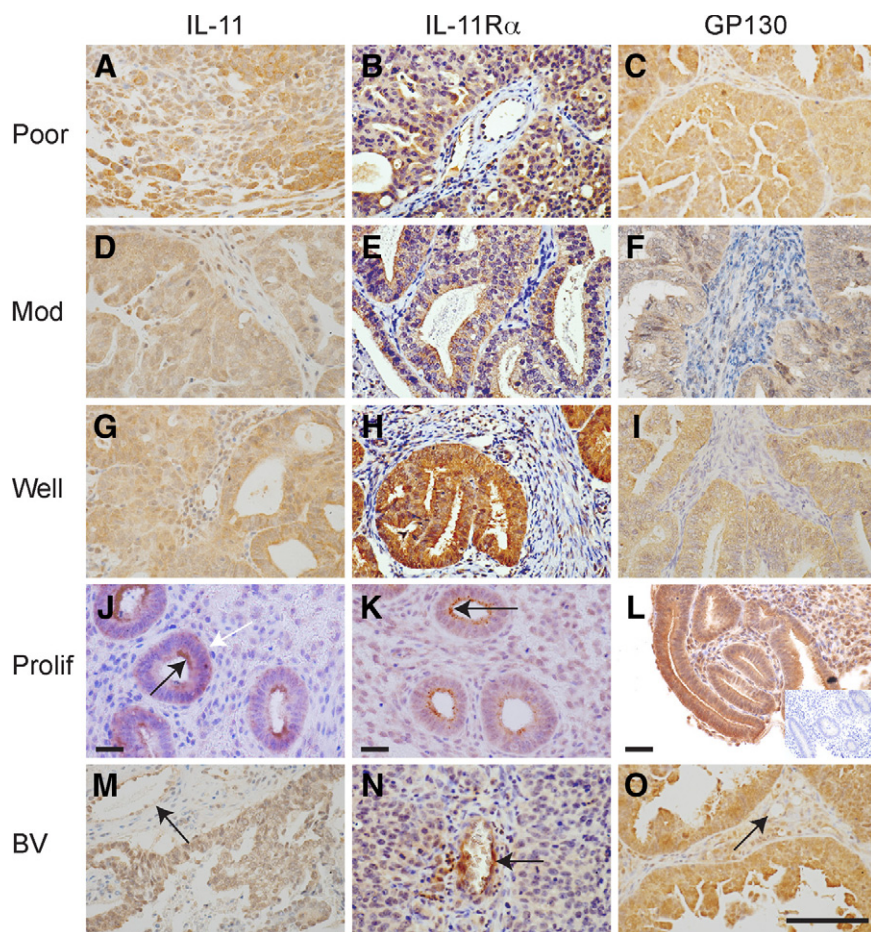


Figure 2. Localization of the site of expression of IL-11, IL-11R α , and GP130 in endometrial adenocarcinomas and normal proliferative endometrium. The site of expression of IL-11 (**A, D, G, J, and M**), IL-11R α (**B, E, H, K, and N**), and GP130 (**C, F, I, L, and O**) in samples of poorly (poor, **A–C**, representative sample 2; Table 1), moderately (mod, **D–F**; representative sample 15; Table 1), and well-differentiated (**G–I**, representative sample 26; Table 1) endometrial adenocarcinomas and proliferative-phase endometrium (**J–L**, representative sample 2; Table 2) by serial section. Intense immunoreactivity as indicated by the brown 3,3'-diaminobenzidine staining was observed to be localized to the glandular epithelial and vascular compartment (BV, **M–O** as indicated by the **arrow**, representative sample 29; Table 1) with diffuse stromal immunoreactivity in all tissue sections with no discernable difference in staining pattern observed within each grade or stage of adenocarcinoma. IL-11 and IL-11R α displayed distinct immunoreactivity on the apical surface in proliferative-phase endometrium (**black arrow** for IL-11 and IL-11R α , **J** and **K**, respectively) with lesser punctuate staining on the basal (**white arrow**, for IL-11, **J**) and lateral surfaces. Control sections were negative for immunoreactivity (**Inset**, representative serial section of **L**, representative sample 2; Table 2). Scale bar = 50 μ mol/L.

basal or lateral surfaces (Figure 2K, representative sample 2; Table 2) with greater intensity of staining observed in the stroma compared with IL-11. GP130 immunoreactivity was localized to the apical and baso-lateral surfaces of the luminal and glandular epithelium with stromal re-

activity, which was greater than that observed for IL-11 or IL-11R α (Figure 2L, representative sample 2; Table 2). Incubating sections with nonimmune IgG from the host species showed no immunoreactivity (inset shown; Figure 2L). Furthermore, the expression of FP receptor (Fig-

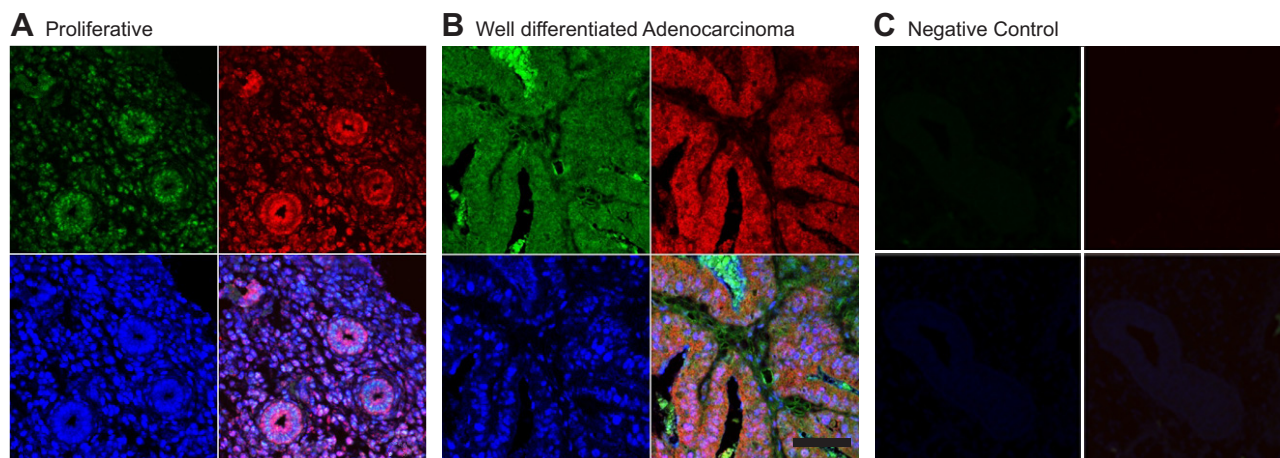


Figure 3. Colocalization of expression of FP receptor, IL-11, and IL-11R α in well-differentiated endometrial adenocarcinoma and proliferative-phase endometrium. Colocalization of the expression of FP receptor (**A and B, lower left panel**, blue channel), IL-11R α (**A and B, upper right panel**, red channel), and IL-11 protein (**A and B, upper left panel**, green channel) colocalized (**A and B, lower right panel**, merged purple channel) in the glandular epithelium and vascular endothelium in proliferative-phase endometrium (**A**, representative sample 9; Table 2) and well-differentiated endometrial adenocarcinoma (**B**, representative sample 27; Table 1) by immunofluorescence. The control section **C** (representative of serial section of representative sample 9; Table 2) was negative for immunoreactivity for all three markers. Scale bar = 50 μ mol/L.

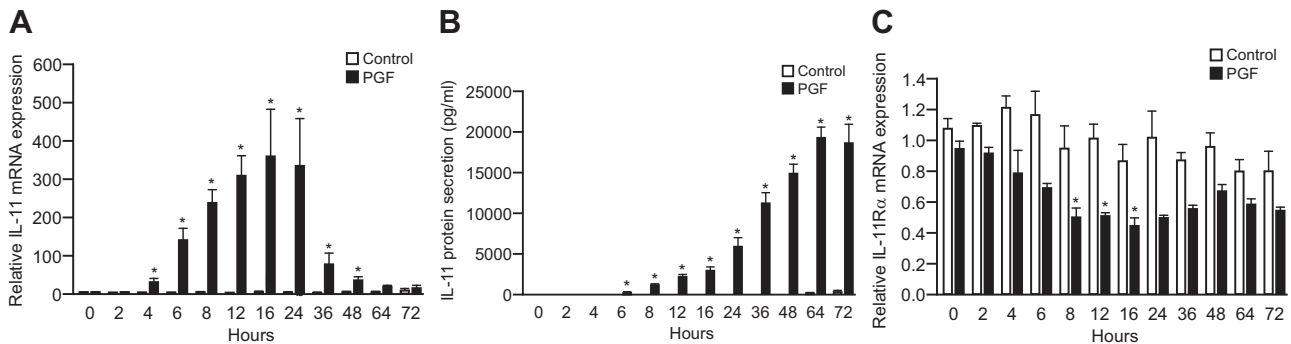


Figure 4. IL-11 and IL-11R α expression in Ishikawa FPS cells in response to 100 nmol/L PGF_{2α}. IL-11 mRNA (**A**) and protein (**B**) expression in Ishikawa FPS cells following treatment of cells for 0, 2, 4, 6, 8, 12, 16, 24, 36, 48, 64, and 72 hours with vehicle or 100 nmol/L PGF_{2α} as measured by real-time quantitative RT-PCR analysis and ELISA, respectively. Data are represented as mean \pm SEM * P < 0.001 for PGF treatment compared with control treatment. **C:** IL-11R α mRNA expression in Ishikawa FPS cells following treatment of cells for 0, 2, 4, 6, 8, 12, 16, 24, 36, 48, 64, and 72 hours with vehicle or 100 nmol/L PGF_{2α} as measured by real-time quantitative RT-PCR analysis. Data are represented as mean \pm SEM * P = 0.04, for 8 hours, and ** P = 0.03 for 12, and 16 hours, for PGF treatment compared with control treatment.

ure 3, A and B, lower left panel, blue channel), IL-11R α (Figure 3, A and B, upper right panel, red channel), and IL-11 protein (Figure 3, A and B, upper left panel, green channel) colocalized (Figure 3, A and B, lower right panel, merged purple channel) in glandular epithelium and vascular endothelium in proliferative-phase endometrium (Figure 3A, representative sample 9; Table 2) and well-differentiated endometrial adenocarcinoma (Figure 3B, representative sample 27, Table 1) by immunofluorescence. The control section (Figure 3C, representative of serial section sample 9; Table 2) was negative for immunoreactivity for all three biomarkers.

PGF_{2α}-FP Receptor Activation Induces IL-11 Expression and Release in Ishikawa FPS Cells

Because FP receptor, IL-11, and IL-11R α colocalized in the glandular epithelium in endometrial adenocarcinomas, PGF_{2α} signaling to IL-11 was investigated using a human endometrioid endometrial adenocarcinoma cell line stably transfected with the FP receptor (Ishikawa FPS cells).¹¹ FPS cells were stimulated with vehicle or 100 nmol/L PGF_{2α} for the times indicated in the figure legend. PGF_{2α} stimulation resulted in a significant time-dependent increase in the relative expression of IL-11 mRNA (Figure 4A, ANOVA, P < 0.001) and protein in FPS cells (Figure 4B, ANOVA, P < 0.001), with maximal responses observed after 16 and 72 hours, respectively. In contrast, PGF_{2α}-FP receptor stimulation of FPS cells promoted a significant time-dependent down-regulation of IL-11R α mRNA expression compared with control (Figure 4C, t -test, P = 0.04, 0.03, and 0.03 for 8, 12 and 16 hours, respectively). No significant alteration in mRNA expression of GP130 was observed in FPS cells in response to PGF_{2α}-FP receptor activation (data not shown).

PGF_{2α}-FP Receptor Activation Induces the Expression of IL-11 via the Calcineurin-NFAT Pathway

The signal transduction pathways mediating PGF_{2α}-FP receptor signaling to IL-11 were investigated. FPS cells

were stimulated with vehicle or 100 nmol/L PGF_{2α} for 24 hours. In parallel, cells were also treated with vehicle or PGF_{2α} in the presence of the FP receptor antagonist AL8810 or chemical inhibitors of G_{q/11} (YM254890), protein kinase (PKC) (RO-318220), PKA (4-cyano-3-methylisoquinoline), calcineurin (cyclosporine A), NFAT (Inca-6), or calcium chelator EGTA. Treatment of FPS cells with PGF_{2α} promoted a significant increase in IL-11 mRNA (Figure 5A, t -test P = 0.01) and protein expression (Figure 5B, t -test P = 0.01). Cotreatment of FPS cells with FP receptor antagonist AL8810 (t -test P = 0.001) or chemical inhibitors of G_{q/11} (YM254890; t -test, P = 0.02), PKC (RO-318220; t -test, P = 0.01), calcineurin (cyclosporine A; t -test P = 0.03), NFAT (Inca-6; t -test, P = 0.03), or calcium chelator EGTA (P = 0.03) but not PKA (4-cyano-3-methylisoquinoline; t -test, P = 0.12) significantly inhibited the expression of IL-11 mRNA (Figure 5A) and release of IL-11 protein (Figure 5B). These data indicate that IL-11 expression and release is regulated via the PGF_{2α}-FP receptor activation of the Gq-PKC-calcium-calcineurin-NFAT signal transduction pathway.

Having identified that the PGF_{2α}-FP receptor activation of IL-11 was mediated via the calcineurin-NFAT signaling pathway, the regulation of IL-11 by the RCAN1, previously known as Down syndrome critical region gene 1 or Adapt 78, which is known to endogenously modulate calcineurin-NFAT signaling²⁵ was explored. RCAN1 isoform 4 (RCAN1-4) is known to bind to calcineurin and inhibit activation of NFAT when overexpressed²⁶ and has been shown by our laboratory to be a negative regulator of chemokine expression.²¹ The temporal regulation of RCAN1-4 in FPS cells by PGF_{2α} was investigated by quantitative RT-PCR and Western blot analysis. Treatment of FPS cells with 100 nmol/L PGF_{2α} resulted in rapid time-dependent induction of RCAN1-4 mRNA (Figure 5C; ANOVA, P < 0.001) and protein (Figure 5D; ANOVA, P < 0.001), with maximal expression after 4 hours with subsequent reduction to basal level. The reciprocal nature of RCAN1-4 expression compared with IL-11 (Figure 4, A and B) suggested that it could be a negative regulator of IL-11 expression. To investigate this, an adenoviral construct expressing the full-length RCAN1-4 cDNA was used. FPS

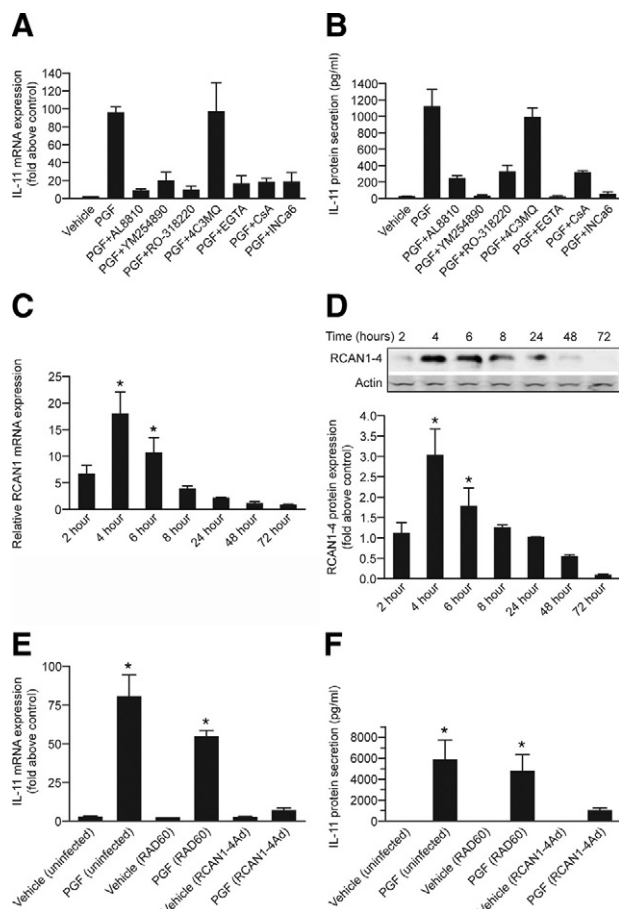


Figure 5. IL-11 is regulated by $\text{PGF}_{2\alpha}$ via the calcium-calcineurin-NFAT pathway. IL-11 mRNA expression (**A**) and protein secretion (**B**) in FPS cells treated for 24 hours, respectively, with vehicle, 100 nmol/L $\text{PGF}_{2\alpha}$ ($P = 0.01$), 100 nmol/L $\text{PGF}_{2\alpha}$ in the absence/presence of AL8810 (50 $\mu\text{mol/L}$; $P = 0.001$), YM254890 (1 $\mu\text{mol/L}$; $P = 0.02$), RO-318220 (1 $\mu\text{mol/L}$; $P = 0.01$), 4-cyano-3-methylisoquinoline (4C3MQ) (1 $\mu\text{mol/L}$; $P = 0.12$), EGTA (1.5 mmol/L; $P = 0.03$), cyclosporine A (CsA) (1 $\mu\text{mol/L}$; $P = 0.03$), or Inca-6 (40 $\mu\text{mol/L}$; $P = 0.03$) as determined by quantitative RT-PCR analysis and ELISA, respectively. Data are represented as mean \pm SEM; PGF treatment is significantly different from PGF and inhibitor treatment or PGF and vehicle control treatment at the previously listed values. RCAN1-4 mRNA (**C**) and protein expression (**D**) in FPS cells treated with 100 nmol/L $\text{PGF}_{2\alpha}$ for 2, 4, 6, 8, 24, 48, and 72 hours as determined by quantitative RT-PCR and Western blot analysis, respectively. Data are represented as mean \pm SEM; $*P < 0.001$ for PGF treatment compared vehicle control treatment at each time point. **E:** FPS cells were infected with either RAD60 control adenovirus or RCAN1-4 adenovirus for 24 hours or left uninfected. Cells were then treated with vehicle or 100 nmol/L $\text{PGF}_{2\alpha}$ for 24 hours and IL-11 mRNA, and protein expression (**F**) was determined by quantitative RT-PCR analysis and ELISA, respectively. Data are represented as mean \pm SEM for PGF treatment compared with vehicle control treatment for mRNA (**E**, $*P = 0.03$) and protein (**F**, $*P = 0.02$).

cells were infected with either a control (RAD60) adenovirus or RCAN1-4 adenovirus for 24 hours before stimulation or left uninfected. Cells were stimulated with vehicle or 100 nmol/L $\text{PGF}_{2\alpha}$ for 24 hours. Overexpression of RCAN1-4 in FPS cells significantly reduced the $\text{PGF}_{2\alpha}$ -FP receptor-mediated induction of IL-11 mRNA expression (Figure 5E; t -test, $P = 0.03$) and protein secretion (Figure 5F; t -test, $P = 0.02$) compared with cells infected with the control virus or control cells, which were uninfected.

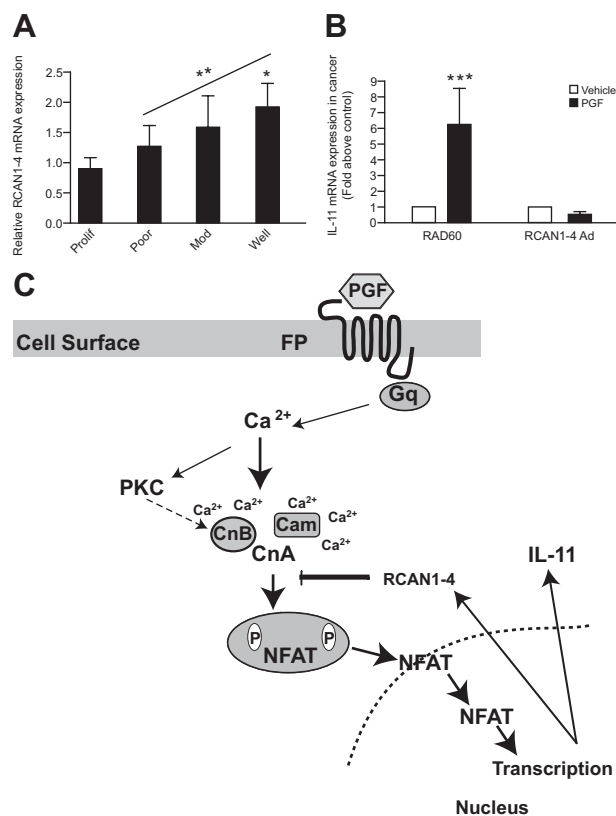


Figure 6. RCAN1-4 expression in endometrial tissues. **A:** Relative mRNA expression of RCAN1-4 in normal proliferative-phase endometrium ($n = 10$) and poorly ($n = 10$), moderately ($n = 10$), and well-differentiated ($n = 10$) endometrial adenocarcinoma as determined by real-time quantitative RT-PCR analysis. Data are represented as mean \pm SEM $*P < 0.03$ for well-differentiated adenocarcinoma compared with proliferative-phase endometrium ANOVA posttest for linear trend between poor, moderate, and well-differentiated adenocarcinomas; $**P = 0.002$. **B:** Poorly differentiated endometrial adenocarcinoma explants (samples 1 to 4; Table 1) were infected with either RAD60 control adenovirus or RCAN1-4 adenovirus for 24 hours and then stimulated with vehicle or 100 nmol/L $\text{PGF}_{2\alpha}$ for 24 hours and subjected to quantitative RT-PCR analysis for IL-11 mRNA expression. Data are represented as mean \pm SEM $***P < 0.003$ for PGF treatment compared with vehicle control treatment. **C:** A schematic summary. $\text{PGF}_{2\alpha}$ -FP receptor activation in endometrial adenocarcinoma cells promotes the induction of RCAN1-4 and IL-11 via the Gq-phospholipase C-PKC-calcium (Ca^{2+})-calcineurin-NFAT cascade. RCAN1-4 is temporally activated in a reciprocal manner to IL-11 by PGF -FP receptor signaling and acts as a negative regulator of the calcineurin pathway to regulate IL-11 expression.

Expression and Regulation of RCAN1-4 in Endometrial Tissues

Because $\text{PGF}_{2\alpha}$ -FP receptor signaling to IL-11 was regulated by RCAN1-4, the mRNA expression of RCAN1-4 (Figure 6A) in human endometrial adenocarcinoma compared with normal proliferative-phase endometrium was investigated by quantitative RT-PCR analysis. RCAN1-4 expression was significantly higher in well-differentiated adenocarcinoma compared with normal proliferative phase endometrium (t -test $P = 0.03$). Interestingly, we observed a significant linear trend for RCAN1-4 mRNA expression, which decreased with increasing tumor grade, such that RCAN1-4 mRNA expression was significantly lower in poorly differentiated endometrial adenocarcinomas (Figure 6A, ANOVA, $*P$ posttest for linear trend, $P = 0.002$).

RCAN1-4 Is a Negative Regulator of IL-11 Expression in Endometrial Adenocarcinoma Explants

Finally, the role of RCAN1-4 as a negative regulator of IL-11 in endometrial adenocarcinomas was investigated *ex vivo*. Four poorly differentiated endometrioid endometrial adenocarcinoma explants (samples 1 to 4; Table 1) were infected with either a control (RAD60) adenovirus or RCAN1-4 adenovirus for 24 hours before stimulation with vehicle or 100 nmol/L PGF_{2α} for 24 hours (Figure 6B). Overexpression of RCAN1-4 in these endometrial adenocarcinoma samples significantly reduced the PGF_{2α}-FP receptor-mediated induction of IL-11 mRNA expression in explants compared with control virus-infected tissue (*t*-test, *P* = 0.003).

Discussion

IL-11 has been shown to regulate cell motility, invasion, and metastasis *in vitro* and *in vivo*.^{15, 20, 27, 28} The present study demonstrated elevated expression of IL-11 in endometrial adenocarcinomas compared with normal endometrium from the proliferative phase of the menstrual cycle. As the endometrium of postmenopausal women is no longer under normal hormonal control, the tissue is atrophic and often not attainable for analysis, we chose normal proliferative-phase endometrium as our comparator. This is the phase of the menstrual cycle that exhibits rapid cellular proliferation, differentiation, and tissue remodeling and is the phase of the menstrual cycle with the highest expression of FP receptor.¹³ Interestingly, the present study highlights a significant variation in expression of IL-11 with grade of adenocarcinomas, with highest levels observed in high-grade (poorly differentiated) adenocarcinomas. Furthermore, IL-11 and IL-11Rα protein expression colocalized with FP receptor expression in normal endometrial tissue and endometrial adenocarcinomas. The pattern of expression of IL-11, IL-11Rα, and its coreceptor GP130 was similar in endometrial adenocarcinoma compared with normal proliferative-phase endometrium in the present study confirming the localization of these biomarkers as reported by others in normal endometrium^{29–32} and other carcinomas.^{33,34} Moreover, we have shown that IL-11 is regulated by PGF_{2α} via the FP receptor in an *in vitro* model system of endometrial adenocarcinoma cells and poorly differentiated endometrial adenocarcinoma explants *ex vivo*. To our knowledge, this is the first study to report on the expression, localization, and cellular mechanism regulating IL-11 in endometrial adenocarcinomas.

Although little is known of the role of IL-11 in endometrial cancer, IL-11 is essential for normal reproduction in the human and mouse.^{35,36} IL-11 and IL-11Rα expression are dysregulated in endometrium of infertile women with endometriosis,^{35,36} and IL-11 in the normal endometrium is known to promote the migration of trophoblast cells.²⁷ IL-11 and IL-11Rα have been shown to correlate with tumor progression, cellular growth and differentiation, and poor prognosis in breast and colorectal can-

cers^{18,19} and is associated with breast cancer cell metastases to bone.¹⁵ To investigate the regulation of IL-11, IL-11Rα, and GP130 in endometrial adenocarcinomas by the FP receptor, we used Ishikawa FPS cells, stably expressing the FP receptor. This *in vitro* approach has previously been used by our laboratory and others to interrogate PGF_{2α}-FP receptor signaling.^{10,11,37–39} Using this model system, we have previously demonstrated that the PGF_{2α}-FP receptor signaling in Ishikawa FPS cells parallels the *ex vivo* effects of PGF_{2α} on endometrial adenocarcinoma explants, indicating that it is a suitable model system to interrogate FP receptor signaling in endometrial epithelial cells *in vitro*.^{10,11}

Here we have demonstrated that PGF_{2α} promotes the synthesis and release of IL-11 in Ishikawa FPS cells in a time-dependent manner. We confirmed that PGF_{2α}-FP receptor signaling regulated IL-11 mRNA expression *ex vivo* using endometrial adenocarcinoma explants. Interestingly, PGF_{2α}-FP receptor interaction promoted a time-dependent decrease in the mRNA expression of IL-11Rα in Ishikawa FPS cells, suggesting that a negative feedback loop was being activated to prevent autocrine induction of the IL-11Rα/GP130 receptor complex by IL-11.

Because expression of IL-11Rα mRNA is significantly reduced in endometrial adenocarcinomas compared with normal proliferative-phase endometrium, it is feasible that activation of the FP receptor in adenocarcinomas *in vivo* could down-regulate expression of the IL-11Rα mRNA in a similar manner to our observation with the Ishikawa FPS cell line *in vitro*. We investigated whether the mechanism of down-regulation of IL-11Rα *in vitro* was mediated via IL-11; however, treatment of FPS cells with the recombinant IL-11 had no effect on IL-11Rα mRNA expression (data not shown), indicating that either PGF_{2α} was having a direct inhibitory effect on IL-11Rα mRNA synthesis or another factor produced by PGF_{2α}-FP receptor signaling was regulating IL-11Rα mRNA in Ishikawa FPS cells *in vitro*.

The intracellular signal transduction pathways mediating the role of the FP receptor in regulating IL-11 was investigated using chemical inhibitors of intracellular signaling. IL-11 was found to be regulated in FPS cells at the mRNA and protein level by PGF_{2α} via the PKC-calcium-calcineurin-NFAT signaling pathway. NFAT activation by calcineurin, which mediates its dephosphorylation and translocation to the nucleus, is known to be regulated by the RCAN.²⁵ RCAN1-4 is known to bind to calcineurin, and previous studies have shown that overexpression of this protein results in an inhibition of calcineurin activation of NFAT.^{26,40,41} The temporal pattern of expression of RCAN1-4 mRNA and protein in FPS cells was investigated in response to PGF_{2α} and found to be regulated in a reciprocal manner to IL-11, reaching a peak of expression that preceded IL-11 × 8 hours, suggesting that RCAN1-4 could act as a negative regulator of IL-11 expression. Indeed, overexpression of RCAN1-4 and subsequent inhibition of NFAT activation significantly inhibited the PGF_{2α}-mediated activation and release of IL-11 from FPS cells. This is in agreement with other published

observations that show that expression of this isoform is induced by NFAT⁴¹ and can negatively regulate chemokines, such as IL-8, in endometrial epithelial cells.²¹

Dysregulated RCAN (also called Down syndrome candidate region 1) expression has been linked to pathological processes. For example, RCAN overexpression in brains of Down syndrome fetuses⁴⁰ and Alzheimer's patients⁴² is thought to enhance neurodegenerative conditions. RCAN expression is known to be up-regulated by glucocorticoids⁴³ and is induced in endothelial cells as a negative feedback regulator of endothelial cell proliferation and branching.^{41,44} We investigated the expression of RCAN1-4 in normal proliferative-phase endometrium and endometrial adenocarcinomas. We found significantly lower RCAN1-4 expression with advancing tumor grade, such that levels of RCAN1-4 were lowest in high-grade (poorly differentiated) adenocarcinomas, the opposite pattern to that seen for IL-11 expression. Furthermore, RCAN1-4 overexpression could negatively regulate IL-11 expression in cancer explants *ex vivo* similarly to our observations using the Ishikawa FPS cell line *in vitro*. Although we found no difference within each grade with respect to FIGO stage, our data show that RCAN1-4 expression reduces coincident with advancing grade of endometrial adenocarcinoma (ie, from well-differentiated or low-grade to poorly differentiated or high-grade cancer). These findings suggest that the natural brake created by RCAN1-4 to control calcineurin signaling and cytokine production is defective in high-grade (poorly differentiated) compared with low-grade (well-differentiated) adenocarcinomas. This results in unopposed PGF_{2α}-FP receptor signaling via the calcium-calcineurin-NFAT pathway to elevate cytokine production such as IL-11 and may confer an advantage to tumor progression and metastasis. However, further studies are needed to determine the prognostic and/or predictive value of IL-11, RCAN1-4, and the calcineurin-NFAT pathway in women with early or advanced endometrial adenocarcinoma.

In conclusion, these data (as summarized in Figure 6C) show that PGF_{2α}-FP receptor activation in endometrial adenocarcinoma cells regulates IL-11 expression via the Gq-phospholipase C-PKC-calcium-calcineurin-NFAT pathway. Moreover, RCAN1-4 was identified as a negative regulator of PGF_{2α}-FP receptor-mediated IL-11 expression in endometrial adenocarcinoma cells stably expressing the FP receptor *in vitro* and tissue explants *ex vivo*. Further studies to evaluate whether RCAN signaling can be exploited therapeutically to negatively control calcineurin-NFAT signaling, and IL-11 production may be of potential clinical significance for women with type 11 high-grade (poorly differentiated) endometrial adenocarcinoma.

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